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Retinoid Activation of Retinoic Acid Receptors but Not of Retinoid X Receptors Promotes Cellular Differentiation and Replication of Human Cytomegalovirus in Embryonal Cells†

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The susceptibility of human embryonal cell line NT-2/D1 to replicate human cytomegalovirus (hCMV) is dependent on retinoic acid (RA) stimulation. Physiological responses to retinoic acid involve two distinct subfamilies of nuclear receptors, the RA receptors (RARs) and retinoid X receptors (RXRs), which function by activating transcription as heterodimeric or RXR homodimeric complexes from *cis*-acting DNA response elements. At present, it is not clear whether the association between these two classes of receptors can lead to multiple distinct induction pathways by signalling one or both receptor partners. Here we have determined, by selectively activating endogenous receptors with novel synthetic ligands specific for either RARs or RXRs, what ligand interaction is physiological in the retinoid receptor pathways necessary for inducing replication of hCMV in differentiated embryonal cells. We show that ligand binding to RAR alone is sufficient and that exclusive ligand activation of RXR is insufficient for inducing replication of hCMV. We also find that differentiation and inhibition of NT-2/D1 cell growth are promoted by compounds that signal the RAR pathway. These results provide direct evidence that RAR ligand-mediated physiological responses are separable and distinct from RXR ligand activation functions. Moreover, our results provide insight into a hormone response pathway for cellular differentiation that might be coopted by hCMV in the host.

Vitamin A and its derivatives (retinoids) control a variety of essential biological functions, including growth, reproduction, and fetal development. Viruses such as human cytomegalovirus (hCMV) may opportunistically use this homeostatic control molecule of cellular differentiation to advance their own replication. Retinoid signals are mediated by specific nuclear receptors that belong to the steroid/thyroid hormone receptor superfamily. The retinoid receptors are grouped into two subfamilies (retinoic acid [RA] receptors [RARs] and retinoid X receptors [RXRs]) which function as ligand-inducible transcription factors by interacting with *cis*-acting DNA acceptor sites as either heterodimeric RXR-RAR or homodimeric RXR complexes (11, 17, 18, 21, 22, 30, 31, 42). These two receptor subfamilies mediate the cellular responses to two distinct RA isomers, all-*trans* RA (ATRA) and 9-*cis* RA (1, 2, 16, 23, 28, 41). Additionally, RXR forms heterodimeric complexes with other members of the receptor family, including thyroid hormone, vitamin D, and peroxisome proliferation receptors (17, 18, 22, 30, 42, 44). The ATRA isomer binds specifically to members of the RAR subfamily, whereas 9-*cis* RA functions as a pan-agonist in that it binds directly to all of the RXR and RAR subtypes (9, 16, 23, 28). The discovery of multiple retinoid receptors and RA isomers raises questions of the potential redundancy and biological roles of the distinct subfamilies. At present, it is not clear whether the association between these two classes of receptors can mediate different induction pathways.

hCMV contains a primary target promoter for RA transcriptional activation (13) and is dependent on RA stimulation for replication in embryonal carcinoma (NT-2/D1) cells (15). In addition, recent experimental evidence indicates that RA can modulate hCMV expression and replication in other cell systems (4, 40). Consequently, this virus provides a particularly useful tool for detecting functional features of retinoid signalling pathways. The effect of RA on the progress of hCMV infection in NT-2/D1 cells is initially determined by the level of transcription from the major immediate-early promoter (MIEP) of the virus (32). This promoter contains RA-responsive elements that function as specific target sites for the direct interaction of RXR-RAR heterodimers (4, 13) and additional *cis*-acting sequences which secondarily respond to RA by derepression (33, 36). Therefore, stimulation of hCMV replication in NT-2/D1 cells most probably arises from a direct effect of RA on the virus as well as being a consequence of the differentiation of the cells. Retinoids may be of significance in the pathogenesis of hCMV as a correlation in the temporal, and spatial patterns of hCMV expression with the physiologic action of RA in both neonates and adults has been noted (7, 13).

NT-2/D1 cells are known to express the two retinoid receptor subfamilies, RAR and RXR (4, 35). In these cells, both of the naturally occurring RA isomers, ATRA (15) and 9-*cis* RA (4), induce replication of hCMV, reduce the proliferative capacity of the cells, and promote cell differentiation. Although ATRA binds only to members of the RAR subfamily, in living cells it can be converted to 9-*cis* RA, which indirectly results in its ability to modulate the functional properties of the RXR subfamily (16, 28). In addition, 9-*cis* RA can bind with high affinity to both RAR and RXR subtypes. Thus, it is not clear which of the three possible ligand-activated receptor pathways

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(RAR, RXR, or RXR-RAR mediated) leads to replication of hCMV and cellular differentiation and growth. Here we have sought to dissect retinoid signalling pathways by relating different synthetic retinoids to the replication of hCMV and cellular differentiation and growth. We find that viral replication in NT-2/D1 cells is stimulated by ligands that signal the activation of the RAR pathway. Compounds that activate the RAR pathway were also found to be sufficient for inducing differentiation and antiproliferation of the embryonal cells, indicating that hCMV exploits a primary retinoid signalling pathway of the cell.

MATERIALS AND METHODS

Retinoids. ATRA was purchased from Sigma. (E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) and (E)-4-[2-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]benzoic acid (3-MeTTNPB) were prepared as previously described (9, 24). 4-[(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)carbonyl]benzoic acid (LG100064) and4-[1-(3-bromo-5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthy)ethenyl]benzoic acid (LG100147) were synthesized as described previously (10). All compounds gave good elemental analyses. The structures of the natural and synthetic retinoids used in this study are shown in Fig. 1. Stock solutions were prepared in dimethyl sulfoxide and/or ethanol and stored under argon at −70°C. Further dilutions were made in Dulbecco's modified Eagle's medium (DMEM) supplemented with charcoal resin-treated fetal bovine serum before use. Handling of retinoids was carried out in subdued lighting, and all containers, tissue culture flasks, and plates containing retinoids were covered with aluminum foil.

Virus and cells. The Towne strain of hCMV was used throughout this study. The virus stock was prepared in human foreskin fibroblasts (HFF) by infection at a low multiplicity. All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of gentamicin per ml.

Plasmids and transfections. The receptor expression vectors pRShRARα (14) and pRShRXRα (28), used in the cotransfection assay, and the reporter plasmids MTV-TREp2-LUC (38), containing two copies of the palindromic thyroid hormone response element, and the CRBPII-tk-LUC (29), carrying the CRBPII (cellular retinol-binding protein II) response element, have been described elsewhere. hCMV MIEP-RARE-tk-LUC, containing an hCMV RA response element of the MIEP (13), was constructed by replacing a *Hin*dIII-*Bgl*II fragment from pRARE(cd) (13) in the tk-LUC vector (29). CV-1 cells were transfected by the calcium phosphate coprecipitation method in 96-well plates for 6 h as described previously (8). In each transfection reaction, 20 μg of DNA per ml of transfection buffer was used. Plasmid ratios used in the specific experiments are indicated in the figure legends. The cells were incubated for approximately 40 h with the different ligands. Control transfections received solvent alone. Cell lysates were prepared as described previously (8) and assayed for luciferase and β-galactosidase activities. Luciferase activity was expressed as the normalized response, which is the luciferase activity divided by the β-galactosidase activity.

Detection and quantification of viral DNA and infectious hCMV. Approximately 2×10^6 NT-2/D1 cells seeded in 25-cm^2 flasks were exposed to the different retinoids diluted in DMEM supplemented with 5% charcoal resin-treated fetal bovine serum for 5 days and then infected with hCMV (Towne strain) at a multiplicity of infection (MOI) of 4. After a 3-h adsorption period, the virus inoculum was removed, the cultures were washed five times with phosphate-buffered saline (PBS), and fresh DMEM supplemented with 3% charcoal resin-treated fetal bovine serum containing the retinoids was added. On days 3 and 6 after infection, the cultures were fed. On various days postinfection, the cells were harvested and frozen in aliquots in liquid nitrogen. Extracellular virus was not detected by plaque assay or fluorescence focus assay in the supernatant of infectious cultures. Total cell DNA was prepared by standard procedures. The DNAs were blotted onto nylon membranes, hybridized with a ^{32}P-labeled probe corresponding to an *Hpa*I-*Stu*I fragment of the UL122 coding region of hCMV, and the amount of hybridized radioactive probe per specific sample was determined by scintillation counting. The assay procedure was validated by treating infected cells with a specific viral DNA inhibitor (foscarnet) which quantitatively inhibited the hybridization signal in a dose-response manner.

Quantitation of infectious particles of hCMV was determined by fluorescence focus assays on HFF. Briefly, the harvested infected NT-2/D1 cells were sonicated and plated at various dilutions onto HFF monolayers. After a 2-h adsorption period, the virus inoculum was removed, cultures were washed five times with PBS, and fresh medium was added. The cultures were allowed to incubate for 24 h at 37°C. After this incubation period, cells were fixed in methanol for 2 min and reacted with hCMV immediate-early monoclonal antibody 810 (dilution of 1:60) for 1 h at 37°C. The bound antibody was determined by indirect immunofluorescence with a secondary antibody, goat anti-mouse fluorescein conjugate (dilution of 1:50; Sigma Immunochemicals).

Differentiation marker analyses. The presence of neurofilament in NT-2/D1 cells treated with the different retinoids was assayed by immunofluorescence microscopy with cells grown on chamber slides and fixed with methanol for 2 min as previously described (6). An antineurofilament (M_r-200,000 polypeptide) rabbit antisera (dilution of 1:100; Chemicon International Inc.) (5) and a goat anti-rabbit fluorescein conjugate (dilution of 1:50; Sigma Immunochemicals) were used. The percentage of cells containing neurofilament was determined by direct counting. Expression of the cell surface antigens (SSEA-3 and A2B5) was studied by fluorescence-activated cell sorter analysis, using established techniques. NT-2/D1 cells were harvested by trypsinization, and approximately 10^6 cells were reacted with an anti-SSEA-3 (dilution of 1:2; Developmental Studies Hybridoma Bank, University of Iowa) (5) or anti-A2B5 (dilution of 1:50; American Type Culture Collection) (5) monoclonal antibody. Cells were then washed three times, stained with fluorescein-conjugated goat anti-mouse secondary antibody, and washed again, and fluorescence was measured by flow cytofluorimetry using a FACScan (Becton Dickinson, Mountain View, Calif.).

Proliferation assays. Following 7 days of retinoid treatment, the NT-2/D1 cells were seeded at a density of 2×10^4 cells per well of 96-well plates. Cells were incubated at 37°C for 16 h with 50 μl of [^{3}H]thymidine (10 μCi/ml; 5 Ci/mmol) in DMEM supplemented with 5% charcoal resin-treated fetal bovine serum.

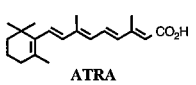
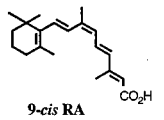
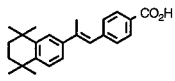
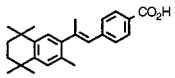
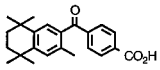
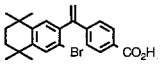
Compound	EC_{50} (nM)	
	RARα	RXRα
 ATRA	352 ± 31	916 ± 70
 9-*cis* RA	191 ± 20	100 ± 25
 TTNPB (RAR-L)	30 ± 6	> 10^4
 3-CH_3-TTNPB (RAR/RXR-L)	340 ± 30	1200 ± 120
 LG100064 (RXRa-L)	> 10^4	379 ± 43
 LG100147 (RXRb-L)	> 10^4	51 ± 6

FIG. 1. Structures and potency of natural and synthetic retinoid compounds. CV-1 cells were cotransfected with 1 μg of pRShRARα or pRShRXRα per ml together with 5 μg of MTV-TREp2-LUC or CRBPII-tk-LUC reporter, respectively, per ml and 5 μg of pRSV-β-gal per ml as an internal control. pGEM4 plasmid DNA (9 μg/ml) was added to bring the final DNA concentration to 20 μg/ml. The cells were incubated for approximately 40 h with increasing concentrations of the indicated ligands. Cell lysates were prepared and assayed for luciferase and β-galactosidase activities. Luciferase activity is expressed as the normalized response, which is the luciferase activity divided by the β-galactosidase activity. The EC_{50} value is defined as the concentration of ligand giving 50% of the maximal observed effect upon transcription of the luciferase reporter. The data shown represent the means ± standard errors of the means of at least three independent EC_{50} determinations. Similar results were obtained in RARβ or -γ and RXRβ or -γ cotransfection experiments.

After this time, cells were washed extensively with PBS and harvested after addition of 50 μl of 3% sodium dodecyl sulfate. The amount of specific thymidine incorporation per well was determined by scintillation counting.

RESULTS

Synthetic RA analogs that are highly selective and potent ligands for activating RAR and RXR subfamilies. Physiological responses to RA involve two distinct subfamilies of nuclear receptors, RARs and RXRs. These receptors activate transcription as RXR homodimeric or heterodimeric complexes that bind to specific sequences at or near promoters. We have synthesized several novel chemical analogs of RA that have the specific property of activating either RARs, RXRs, or both, as shown in the following set of experiments (Fig. 1). We measured the selectivity of these compounds to activate RAR- or RXR-mediated transcription by a coupled plasmid transfection assay (Fig. 1). In this assay, we introduced an expression vector for the indicated retinoid receptor along with a reporter construct carrying either the thyroid hormone response element (TRE-pal-RARE) or CRBPII (CRBPII-RXRE) (1). The TRE-pal-RARE reporter has been shown to be dependent on cotransfection of RAR expression vectors for activation, while the reporter CRBPII-RXRE has been demonstrated to be exclusively activated by and dependent on cotransfection of RXR expression vectors (29, 45). Therefore, this coupled plasmid transfection assay is well suited for determining the selectivity and potency of compounds that activate RAR or RXR transcriptional pathways. The potency (concentration of retinoid that produced 50% of the maximal response [EC_{50}]) of the retinoids was determined by varying their concentration over 8 orders of magnitude (from 10^{-12} to 10^{-5} M). As expected, ATRA, 9-*cis* RA, and the pan-agonist 3-MeTTNPB were capable of inducing reporter activity mediated by both RAR and RXR induction pathways (Fig. 1). As previously reported (16, 28), the RAR-selective ligand (TTNPB) was shown to be a potent activator of RAR (EC_{50} of 30 nM) and was unable to effect activation of RXR (Fig. 1). By contrast, LG100064 and LG100147 selectively activated RXR with EC_{50} values of 379 and 51 nM, respectively (Fig. 1). These data demonstrate the RAR selectivity of TTNPB and the RXR selectivity of LG100064 and LG100147. Importantly, the use of these metabolically stable compounds provides a unique tool to modulate, in the cell, distinct retinoid signalling pathways.

Exclusive induction of the RAR pathway is sufficient for activating the cytomegalovirus RA response element. Previously we found that the MIEP of hCMV contains an RA response element (13). This element is composed of two direct repeat motifs separated by five nucleotides and is activated by the binding of endogenous RAR-RXR heterodimers (4, 13). Because RAR alone is unable to bind the viral RA response element with high affinity (4), these studies demonstrate that NT-2/D1 cells express a functional retinoid receptor complex of both RARs and RXRs, which can be transactivated by RA. In the following experiments, we use the synthetic retinoid compounds characterized above to demonstrate what retinoid signalling pathway is operative in activation of the viral RA response element. The assay used a plasmid construct that contained the hCMV MIEP RA response element fused to a luciferase reporter gene (13). The reporter gene activities measured when various synthetic retinoids were added to the transfected cells are shown in Fig. 2. The viral reporter was activated by both 3-MeTTNPB and TTNPB but not by the RXR-selective compounds LG100064 and LG100147 (Fig. 2). Cotransfection of expression vectors for RAR, RXR, or both yielded the same dose-response curves for each of the ligands

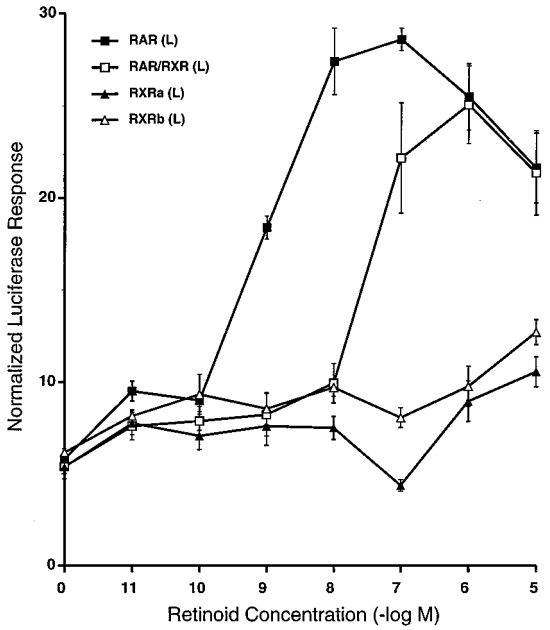


FIG. 2. Transactivation of the hCMV MIEP RA response element by synthetic retinoids. CV-1 cells were cotransfected with 5 μg of hCMV MIEP-RARE-tk-LUC reporter per ml together with 5 μg of pRSV-β-gal per ml and pGEM4 to bring the final plasmid concentration to 20 μg/ml. The cells were incubated for approximately 40 h with increasing concentrations of TTNPB [RAR (L)], 3-MeTTNPB [RAR/RXR (L)], L100064 [RXRa (L)], or LG100147 [RXRb (L)]. The data are presented as normalized responses calculated as indicated in the legend to Fig. 1. Data shown represent the means ± the standard errors of the means of triplicate determinations. Similar results were obtained in cotransfection experiments with RARα or -β or RXRα and in dual cotransfection assays with RARα or -β and RXRα except that the observed normalized response was enhanced.

(4). These experiments reveal the potency of TTNPB in RAR activation of the viral response element and the inability of RXR to contribute exclusively to ligand activation, suggesting a restricted dependence of the hCMV promoter element on RAR transactivation functions. These results (Fig. 1 and 2) are in agreement with those of previously published cotransfection assays indicating that RAR and RXR may differentially mediate distinct retinoid induction pathways (11, 12, 17, 18, 20–22, 25–27, 30, 31, 39, 42, 44). Importantly, one can specifically address how these retinoids stimulate hCMV replication in NT-2/D1 cells, and thus this model provides a unique opportunity for directly evaluating the physiological response of the different retinoid receptor pathways.

hCMV replication in NT-2/D1 cells requires activation of the RAR pathway but fails to replicate upon exclusive stimulation of the RXR pathway. The replication of hCMV in NT-2/D1 cells has been shown to be dependent on RA stimulation (15). Accordingly, we sought to determine whether the RAR pathway characterized above for the viral RA response element is also necessary for inducing replication of hCMV. In the following set of experiments, retinoid stimulation of hCMV was monitored by examining viral replication in infected NT-2/D1 cells in the presence of the different synthetic compounds. Viral DNA replication was determined by slot blot analysis at different times postinfection. As expected, hCMV failed to replicate with solvent alone, but in the presence of ATRA, a dramatic induction (~10^4 genome equivalents per cell) of viral replication was exhibited (Fig. 3). Similarly, treatment of NT-2/D1 cells (which coexpress RAR and RXR sub-

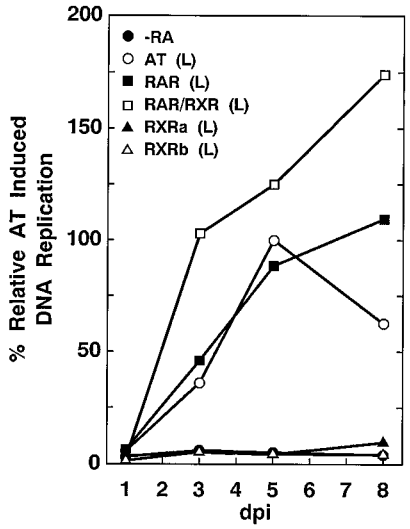


FIG. 3. Selective activation of hCMV DNA replication by different retinoids. NT-2/D1 cells were exposed to ATRA at 10^{-5} M [AT (L)], TTNPB at 10^{-7} M [RAR (L)], 3-MeTTNPB at 10^{-6} M [RAR/RXR (L)], LG100064 at 10^{-6} M [RXRa (L)], LG100147 at 10^{-6} M [RXRb (L)], and vehicle (-RA). Subsequently, cells were infected with hCMV (Towne) at an MOI of 4. At various days postinfection (dpi), the cells were harvested and viral DNA was quantified as described in Materials and Methods. The results, normalized to the number of cells, are expressed as the amounts (percentages) of viral DNA replication relative to the maximum level of newly replicated viral DNA detected in NT-2/D1 cells treated with ATRA (100%). The data represent average values from two independent experiments.

families [4, 35]) with the pan-agonist 3-MeTTNPB also led to replication of hCMV (Fig. 3), with a half-maximal induction observed at ~50 nM (Fig. 4). It should be noted that hCMV replication was not induced to a significantly higher level by

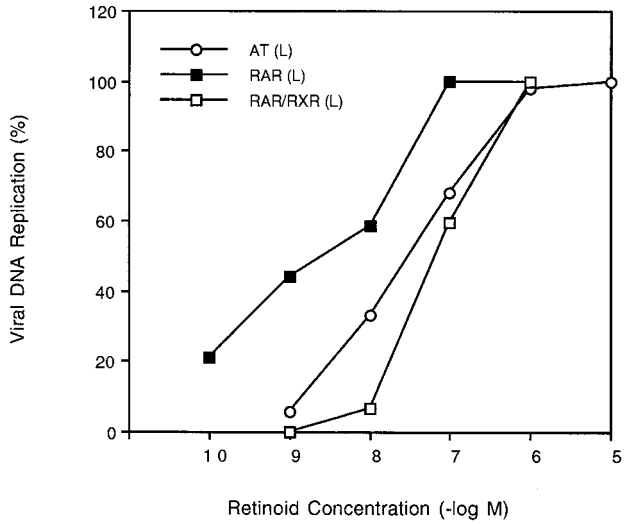


FIG. 4. Potency of different retinoids on the activation of hCMV DNA replication. NT-2/D1 cells were treated with ATRA [AT (L)], TTNPB [RAR (L)], and 3-MeTTNPB [RAR/RXR (L)] at the indicated concentrations. After retinoid induction, cells were infected (at an MOI of 4) with hCMV (Towne) and tested for the presence of viral DNA on day 5 postinfection as described in Materials and Methods. The results, normalized to the number of cells, are expressed as percentages of the amounts (counts per minute) of viral DNA replication relative to the maximum level detected for NT-2/D1 cells treated with each retinoid. Results of one set of the duplicate replication assays are shown.

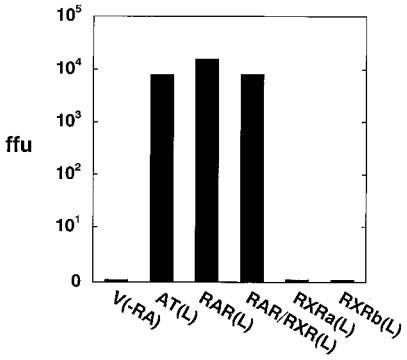


FIG. 5. Production of infectious hCMV in NT-2/D1 cells treated with the different retinoids. NT-2/D1 cells were exposed to ATRA at 10^{-5} M [AT (L)], TTNPB at 10^{-7} M [RAR (L)], 3-MeTTNPB at 10^{-6} M [RAR/RXR (L)], LG100064 at 10^{-6} M [RXRa (L)], LG100147 at 10^{-6} M [RXRb (L)], and the vehicle (-RA). The retinoid-induced cells were infected with the Towne strain of hCMV (at an MOI of 4) and harvested on day 8 postinfection. Infectious virus present in the infected NT-2/D1 cells was determined by fluorescence focus assay on HFF. The number of fluorescence focus units (ffu) of hCMV was determined as described in Materials and Methods. Data shown correspond to a representative example of three independent experiments.

3-MeTTNPB than by ATRA under the conditions of these NT-2/D1 cell infection experiments.

To examine whether an uncoupled activation of RAR and RXR can lead to hCMV replication, NT-2/D1 cells were treated with either TTNPB or LG100064. The RAR-selective ligand (TTNPB) induced viral DNA replication to levels (~10^4 genome equivalents per cell) comparable to those found for ATRA, thus directly implicating RAR activation functions in hCMV replication (Fig. 3). Half-maximal induction of hCMV replication was observed at ~5 nM for TTNPB (Fig. 4), in agreement with the EC_{50} value observed in the retinoid-induced transactivation assays (Fig. 1 and 2). In marked contrast, two structurally dissimilar compounds (LG100064 and LG100147) with similar activities for RXR activation failed to yield significant viral replication (<1 genome equivalent per cell), indicating that ligand binding to RAR alone is sufficient, and exclusive ligand activation of RXR pathways is insufficient, for inducing hCMV replication (Fig. 3).

Further, to determine whether induction of the RAR pathway also leads to production of infectious virus in NT-2/D1 cells, infectious fluorescence focus assays were performed. The results of these experiments (Fig. 5) demonstrate that exposure of cells to ligands that activate RAR permitted production of infectious viral particles (~10^4 fluorescence focus units), while cells treated with the RXR-selective ligands remained totally nonpermissive for viral growth. We conclude from these data that the ligand-induced RAR pathway, but not the RXR pathway, is sufficient for mediating the retinoid induction of hCMV replication.

The RAR pathway is sufficient for retinoid-induced differentiation and antiproliferation of NT-2/D1 cells. Retinoids control a variety of biological processes, including differentiation and proliferation of cells. NT-2/D1 cells differentiate into cell types of neuronal lineage and retard their growth in the presence of RA. Thus, the replication competence of hCMV in RA-treated NT-2/D1 cells may be a consequence of retinoid-induced differentiation of cells and/or reduced proliferative capacity. Alternatively, RA may directly induce viral replication and also initiate cell differentiation and/or antiproliferation. It is possible that distinct retinoid receptor pathways are used for viral replication, differentiation, and antiproliferation

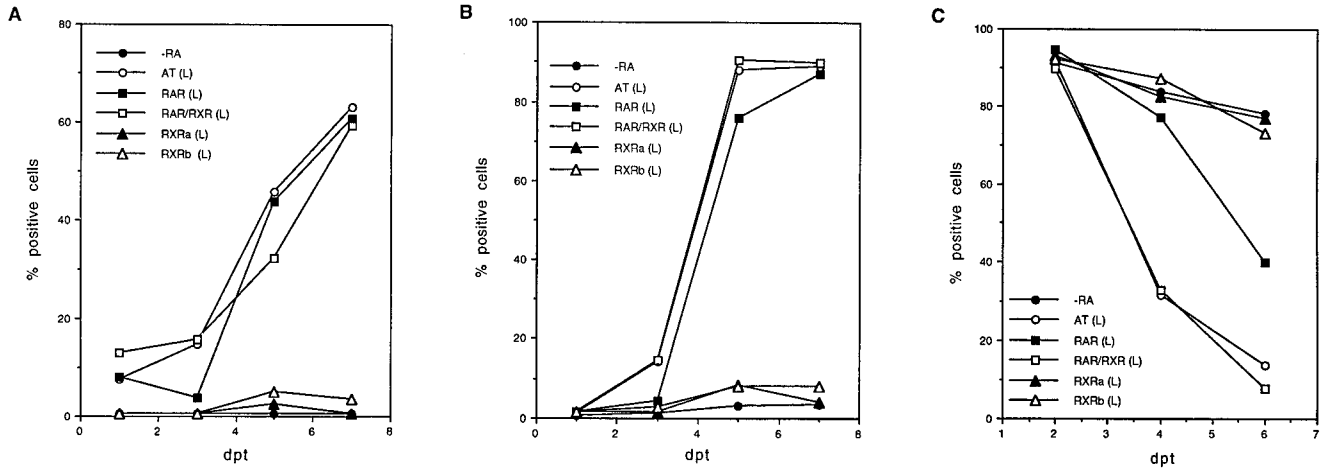


FIG. 6. Expression of neurofilament and surface antigens SSEA-3 and A2B5 in NT-2/D1 cells exposed to different retinoids. NT-2/D1 cells were treated with ATRA at 10^{-5} M [AT (L)], TTNPB at 10^{-6} M [RAR (L)], 3-MeTTNPB at 10^{-6} M [RAR/RXR (L)], LG100064 at 10^{-6} M [RXRa (L)], LG100147 at 10^{-6} M [RXRb (L)], or the vehicle (-RA). At various days after retinoid treatment (dpt), the cultures were either fixed and tested by immunofluorescence microscopy for neurofilament positive cells (A) or trypsinized and assayed by flow cytofluorimetry for the expression of A2B5 (B) and of the SSEA-3 (C) antigens, using the corresponding antibodies as indicated in Materials and Methods. Results of one set of the duplicate immunofluorescence assays are shown.

or that their retinoid signalling pathways converge. If the virus uses a retinoid signalling pathway different from that of differentiation and proliferation, then this would indicate a direct effect of RA on hCMV. On the other hand, if the cellular responses (differentiation and proliferation) to RA parallel those to hCMV replication, then this might indicate an indirect effect. However, in this case, when all pathways converge, then one cannot formally exclude a direct involvement of RA with hCMV. In the following set of experiments, we sought to determine what ligand receptor interaction is required for mediating the cellular responses to RA by exposing NT-2/D1 cells at various times to the different synthetic ligands and analyzing them for their state of differentiation and growth.

Differentiation of NT-2/D1 cells following exposure to RA is indicated by the appearance of the surface marker A2B5 and neurofilament proteins, which are characteristic of the differentiated cells, and the reduction of expression of the cell surface antigen SSEA-3, characteristic of the embryonal carcinoma cells (5). As shown in Fig. 6, cells treated with ligands that activate RAR expressed neurofilament proteins (approximately 60%; Fig. 6A) and induced A2B5 (approximately 90%; Fig. 6B), while cells treated with RXR-exclusive ligands failed to express either neurofilament (less than 5% of the cell population; Fig. 6A) or A2B5 (less than 10% of the cell population; Fig. 6B). Accordingly, after 6 days of treatment with ATRA and 3-MeTTNPB, approximately 90% of cells ceased to express the SSEA-3 antigen. Treatment of the NT-2/D1 cells with the RAR-selective ligand TTNPB for 6 days caused the disappearance of ~60% of the SSEA-3 marker, while no reduction in SSEA-3 expression resulted from exposure to the RXR-selective ligands. We also studied the expression of the Hox 2.9 homeobox gene, which is known to be rapidly and specifically induced by RA in NT-2/D1 cells (37). In agreement with the differentiation data, we also observed the requirement of RAR but not exclusive RXR activation of Hox 2.9 expression after exposure to retinoids (3).

Finally, to determine whether RAR activation also leads to an antiproliferative response, NT-2/D1 cells were exposed to the different synthetic RA analogs and examined for their state of proliferation. Ligands that activate RAR inhibited efficiently the proliferation of NT-2/D1 cells (Fig. 7). By contrast, ligands

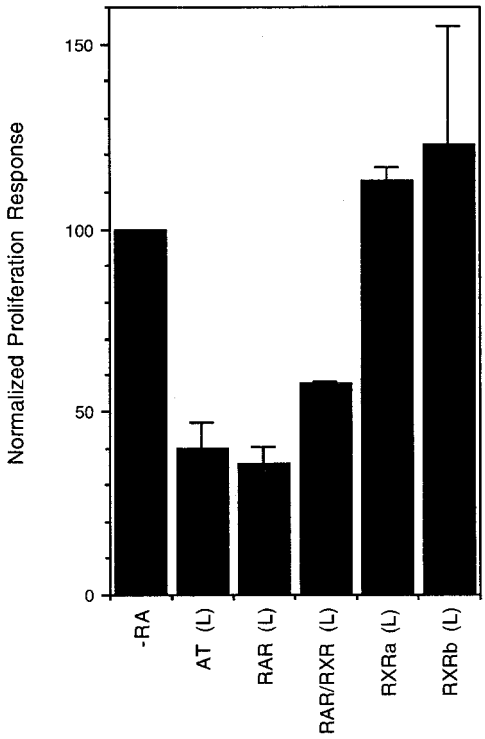


FIG. 7. Effect of different retinoids on NT-2/D1 cell proliferation. NT-2/D1 cells that were exposed to ATRA at 10^{-5} M [AT (L)], TTNPB at 10^{-6} M [RAR (L)], 3-MeTTNPB at 10^{-6} M [RAR/RXR (L)], LG100064 at 10^{-6} M [RXRa (L)], LG100147 at 10^{-6} M [RXRb (L)], or the vehicle (-RA) were seeded at a density of 2×10^4 cells per well in 96-well plates and assayed for the capability to incorporate [^{3}H]thymidine during 16 h as an index of proliferation. The results are expressed as the normalized proliferation response, which is the percentage of proliferation developed by cells treated with the different retinoids relative to the proliferation of cells that received solvent alone. The data shown represent the means of two independent experiments.

that exclusively activate RXR failed to slow the growth rate of this cell line (Fig. 7). Thus, in NT-2/D1 cells, retinoid control of proliferation also appears to depend on RAR transactivation functions.

Together, these experiments indicate that cells treated with ligands that activate RAR promote differentiation and antiproliferation, while exclusive activation of RXR is not sufficient to effect a response. Thus, the observed divergent effects of RAR and RXR ligand-activated pathways are not unique to the virus and might indicate a requirement for cellular differentiation in viral replication.

DISCUSSION

Here we show, by using novel synthetic retinoids, that ligand activation of the RAR pathway but not the interconnected RXR pathway is a prerequisite to replicate hCMV in NT-2/D1 cells. Concordant with viral growth, we also find that differentiation and antiproliferation of NT-2/D1 cells are induced by compounds that mediate RAR transcriptional activation but not by compounds that signal RXR activation. We interpret this result to mean that the RXR pathway is not required in these cells for RA-mediated differentiation and growth processes. These findings have important implications for understanding the action of retinoids relevant to viral biology and hormone receptor function.

In humans, reactivation of hCMV may be frequent and, depending on the immune status of the individual, may or may not be associated with disease. Vitamin response pathways represent one of perhaps many different physiological stimuli that might influence viral activation. Previously, we have speculated on the possible involvement of vitamin A (RA) as a potential modulator of hCMV replication in vivo (see the introduction and reference 13). Although it remains to be determined whether retinoids are used by hCMV in humans, the identification of the RAR pathway in this study is an important step in defining a molecular pathway used by the cell which is coopted by hCMV for its replication. The question of direct activation of hCMV by retinoids is complicated, since the slow growth kinetics of hCMV coincide with the differentiation of the cells by retinoids. Experiments examining differentiation markers revealed that RA-induced differentiation of NT-2/D1 cells requires only RAR activation functions, indicating that these effects are not virus specific and may be indirect. However, consistent with a direct effect and in agreement with the viral replication data, we find that the MIEP of hCMV is selectively activated by RAR through the RAR-RXR heterodimer complex. Further, the role of the RAR-selective pathway for hCMV replication does not appear to be dependent on differentiation or restricted to NT-2/D1 cells, as our recent experimental evidence indicates that RAR but not RXR activation can lead to enhanced replication in HFF (4). In addition, retinoids have been shown to up-regulate hCMV expression in latently infected glioblastoma (U138-MG) cells (40). Combined, these observations lend support to both a direct and an indirect effect of RA action on hCMV. In the case of NT-2/D1 cells, a plausible explanation for our observed results is that RA directly activates MIEP transcription and induces (represses) the expression of a select subset of cellular genes via RAR-RXR heterodimers. These induced (suppressed) gene products, in combination with direct activation by the retinoid receptors, stimulate differentiation and hCMV expression, leading to viral replication in the differentiated cells. Regardless of the underlying mechanism responsible for the replication potential of hCMV in NT-2/D1 cells, the observed viral replication and nonreplication mediated by RAR and RXR pathways, respectively, demonstrate a dramatic functional difference between the interconnected retinoid receptor pathways.

RA is required for growth, morphogenesis, differentiation, and homeostasis (see the introduction). In addition to viral replication, our findings indicate that the activation of RAR alone is sufficient for inducing cellular differentiation and cessation of cell proliferation. While previous studies have established that RAR functions as a heterodimeric complex with RXR (12, 20, 25, 26, 44), our results clearly document that the biological activation function of RAR is separable from that of RXR. The precise mechanism by which RAR but not RXR exclusively affects activation functions in RXR-RAR heterodimeric complexes remains to be elucidated. The lack of activity of the RXR component of RXR–COUP-TF and RXR-thyroid hormone receptor heterodimers may involve similar mechanisms (17, 30, 34). A recent study has indicated that the polarity of RXR-RAR heterodimers directs ligand binding and sensitivity of transactivation (19). In this study, the binding polarity of heterodimers formed by RXR with RAR on direct repeats spaced by five nucleotides was shown to be fixed in 5′-RXR-RAR-3′ orientation (19, 43), and ligand bound exclusively to the downstream receptor (19). In this connection, the hCMV RA response element has an identical spacer length and is selectively bound by heterodimers that are likely to be fixed in 5′-RXR-RAR-3′ orientation. Therefore, our observations are in good agreement with, and most likely related to, the binding polarity of heterodimers formed by RXR and RAR. Combined with many other studies (1, 2, 11, 12, 16–18, 20–23, 25–31, 39, 41, 42, 44, 45), our findings support the notion that specificity in retinoid receptor function is achieved by not only selective DNA recognition of distinct response elements but also selective ligand-mediated activation functions.

In summary, our results document the essential role of only one partner (RAR) within heterodimeric (RXR-RAR) complexes in mediating activation functions and susceptibility of NT-2/D1 cells to hCMV replication. These observations are relevant for understanding the role of the receptor subfamilies in the complex biology of retinoid action and also for providing insight into the regulation of an important human pathogen, hCMV.

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REFERENCES

1. **Allegretto, E. A., M. R. McClurg, S. B. Lazarchik, D. L. Clemm, S. A. Kerner, M. G. Elgot, M. F. Boehm, S. K. White, J. W. Pike, and R. A. Heyman.** 1993. *Trans*activation properties of retinoic acid and retinoic X receptors in mammalian cells and yeast. Correlation with hormone binding and effects of metabolism. J. Biol. Chem. **268:**26625–26633.
2. **Allenby, G., M. T. Bocquel, M. Saunders, S. Kazmer, J. Speck, M. Rosenberger, A. Lovey, P. Kastner, J. F. Grippo, and P. Chambon.** 1993. Retinoic acid receptors and retinoic X receptors: interactions with endogenous retinoic acids. Proc. Natl. Acad. Sci. USA **90:**30–34.

3. **Angulo, A., T. Ogura, R. Heyman, R. Evans, and P. Ghazal.** Unpublished data.
4. **Angulo, A., C. Suto, R. Heyman, and P. Ghazal.** Receptor selective transcriptional and viral replication responses to retinoic acid mediated by a set of polymorphic DR2 and DR5 elements in the cytomegalovirus enhancer. Submitted for publication.
5. **Andrews, P. W., E. Gönczöl, S. A. Plotkin, M. Dignazio, and J. W. Oosterhuis.** 1986. Differentiation of TERA-2 human embryonal carcinoma cells into neurons and HCMV permissive cells. Differentiation **31:**119–126.
6. **Baracchini, E., E. Glezer, K. Fish, R. M. Stenberg, J. A. Nelson, and P. Ghazal.** 1992. An isoform variant of the cytomegalovirus immediate-early auto repressor functions as a transcriptional activator. Virology **188:**518–529.
7. **Baskar, J., P. Smith, G. Cement, S. Hoffman, C. Tucker, S. Kruschke, J. A. Nelson, and P. Ghazal.** Unpublished data.
8. **Berger, T. S., Z. Parandoosh, B. W. Perry, and R. B. Stein.** 1992. Interaction of glucocorticoid analogues with the human glucocorticoid receptor. J. Steroid Biochem. Mol. Biol. **41:**733–738.
9. **Boehm, M. F., M. R. McClurg, C. Pathirana, D. Mangelsdorf, S. K. White, J. Hebert, D. Winn, M. E. Goldman, and R. A. Heyman.** 1994. Synthesis of high specific activity [^{3}H]-9-cis-retinoic acid and its application for identifying unusual binding properties. J. Med. Chem. **37:**408–414.
10. **Boehm, M. F., L. Zhang, B. A. Badea, S. K. White, D. E. Mais, E. Berger, C. M. Suto, M. E. Goldman, and R. A. Heyman.** 1994. Synthesis and structure-activity relationships of novel retinoid X receptor selective retinoids. J. Med. Chem. **37:**2930–2941.
11. **Bugge, T. H., J. Pohl, O. Lonnoy, and H. G. Stunnenberg.** 1992. RXRα, a promiscuous partner of retinoic acid and thyroid hormone receptors. EMBO J. **11:**1409–1418.
12. **Durand, B., M. Saunders, P. Leroy, M. Leid, and P. Chambon.** 1992. All-trans and 9-cis retinoic acid induction of CRABPII transcription is mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs. Cell **71:**73–85.
13. **Ghazal, P., C. DeMattei, E. Giulietti, S. A. Kliewer, K. Umesono, and R. M. Evans.** 1992. Retinoic acid receptors initiate induction of the cytomegalovirus enhancer in embryonal cells. Proc. Natl. Acad. Sci. USA **89:**7630–7634.
14. **Giguère, V., E. S. Ong, P. Segui, and R. M. Evans.** 1987. Identification of a receptor for the morphogen retinoic acid. Nature (London) **330:**624–629.
15. **Gönczöl, E., P. W. Andrews, and S. A. Plotkin.** 1984. Cytomegalovirus replicates in differentiated but not undifferentiated human embryonal carcinoma cells. Science **224:**159–161.
16. **Heyman, R. A., D. J. Mangelsdorf, J. A. Dyck, R. B. Stein, G. Eichele, R. M. Evans, and C. Thaller.** 1992. 9-Cis retinoic acid is a high affinity ligand for the retinoid X receptor. Cell **68:**397–406.
17. **Kliewer, S. A., K. Umesono, R. A. Heyman, D. J. Mangelsdorf, J. A. Dyck, and R. M. Evans.** 1992a. Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. Proc. Natl. Acad. Sci. USA **89:**1448–1452.
18. **Kliewer, S. A., K. Umesono, D. J. Mangelsdorf, and R. M. Evans.** 1992b. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. Nature (London) **355:**446–449.
19. **Kurokawa, R., J. Direnzo, M. Boehm, J. Sugarman, B. Gloss, M. G. Rosenfeld, R. Heyman, and C. K. Glass.** 1994. Receptor polarity and allosteric control of ligand binding dictate the transcriptional responses to retinoic acid. Nature (London) **371:**528–531.
20. **Lehmann, J. M., L. Jong, A. Fanjul, J. F. Cameron, X.-P. Lu, P. Haefner, M. I. Dawson, and M. Pfahl.** 1992. Retinoids selective for retinoid X receptor response pathways. Science **258:**1944–1946.
21. **Leid, M., P. Kastner, and P. Chambon.** 1992. Multiplicity generates diversity in the retinoic acid signalling pathways. Trends Biochem. Sci. **17:**27–433.
22. **Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J.-Y. Chen, A. Staub, J.-M. Garnier, S. Mader, and P. Chambon.** 1992. Purification, cloning, and RXR identity of the Hela cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell **68:** 377–395.
23. **Levin, A. A., L. J. Sturzenbecker, S. Kazmer, T. Bosakowski, C. Huselton, G. Allenby, J. Speck, C. Kratzeisen, M. Rosenberger, A. Lovey, and J. F. Grippo.** 1992. 9-Cis retinoic acid stereoisomer binds and activates the nuclear receptor RXRα. Nature (London) **355:**359–361.
24. **Loelinger, P., W. Bollag, and H. Mayer.** 1980. Arotinoids, a new class of highly active retinoids. Eur. J. Med. Chem. **15:**9–15.
25. **Mader, S., J. Y. Chen, Z. Chen, J. White, P. Chambon, and H. Gronemeyer.** 1993. The patterns of binding of RAR, RXR and TR homo- and heterodimers to direct repeats are dictated by the binding specificities of the DNA binding domain. EMBO J. **12:**5029–5041.
26. **Mader, S., P. Leroy, J.-Y. Chen, and P. Chambon.** 1993. Multiple parameters control the selectivity of nuclear receptors for their response elements: selectivity and promiscuity in response element recognition by RARs and RXRs. J. Biol. Chem. **268:**591–600.
27. **Mangelsdorf, D. J., U. Borgmeyer, R. A. Heyman, J. Y. Zhou, E. S. Ong, A. E. Oro, A. Kakizuka, and R. M. Evans.** 1992. Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. Genes Dev. **6:**329–344.
28. **Mangelsdorf, D. J., E. S. Ong, J. A. Dyck, and R. M. Evan.** 1990. A nuclear receptor that identifies a novel retinoic acid response pathway. Nature (London) **345:**224–229.
29. **Mangelsdorf, D. J., K. Umesono, S. A. Kliewer, U. Borgmeyer, E. S. Ong, and R. M. Evans.** 1991. A direct repeat in the cellular binding protein type II gene confers differential regulation by RXR and RAR. Cell **66:**555–561.
30. **Marks, M. S., P. L. Hallenbeck, T. Nagata, J. H. Segars, E. Appella, V. M. Nikodem, and K. Ozato.** 1992. H-2RIIBP (RXRβ) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. EMBO J. **11:**1419–1435.
31. **Näär, A. M., J.-M. Boutin, S. M. Lipkin, V. C. Yu, J. M. Holloway, C. K. Glass, and M. G. Rosenfeld.** 1991. The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. Cell **65:**1267–1279.
32. **Nelson, J. A., and M. Groudine.** 1986. Transcriptional regulation of the human cytomegalovirus major immediate-early gene is associated with induction of DNase I-hypersensitive sites. Mol. Cell. Biol. **6:**452–461.
33. **Nelson, J. A., C. Reynolds-Kohler, and B. Smith.** 1987. Negative and positive regulation by a short segment in the 5′-flanking region of the human cytomegalovirus major immediate-early gene. Mol. Cell. Biol. **7:**4125–4129.
34. **Schräder, M., K. M. Müller, S. Nayeri, J.-P. Kahlen, and C. Carlberg.** 1994. Vitamin D_3-thyroid hormone receptor heterodimer polarity directs ligand sensitivity of *trans*activation. Nature (London) **370:**382–386.
35. **Segars, J. H., T. Nagata, V. Bours, J. A. Medin, G. Franzoso, J. C. G. Blanco, P. D. Drew, K. G. Becker, J. An, T. Tang, D. A. Stephany, B. Neel, U. Siebenlist, and K. Ozato.** 1993. Retinoic acid induction of major histocompatibility complex class I in NTera-2 embryonal carcinoma cells involves induction of NF-κB (p50-p65) and retinoic acid receptor β-retinoid X receptor β heterodimers. Mol. Cell. Biol. **13:**6157–6169.
36. **Shelbourn, S. L., S. K. Kothari, J. G. P. Sissons, and J. H. Sinclair.** 1989. Repression of human cytomegalovirus gene expression associated with a novel immediate early regulatory region. Nucleic Acids Res. **17:**9165–9171.
37. **Simeone, A., D. Acampora, L. Arcioni, P. W. Andrews, E. Boncinelli, and F. Mavilio.** 1990. Sequential activation of HOX2 homeobox genes by retinoic acid in human embryonal carcinoma cells. Nature (London) **346:**763–766.
38. **Umesono, K., V. Giguère, C. K. Glass, M. G. Rosenfeld, and R. M. Evans.** 1988. Retinoic acid and thyroid hormone induce gene expression through a common responsive element. Nature (London) **336:**262–265.
39. **Umesono, K., K. K. Murakami, C. C. Thompson, and R. M. Evans.** 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell **65:**1255–1266.
40. **Wolff, D., C. Sinzger, P. Drescher, G. Jahn, and B. Plachter.** 1994. Reduced levels of IE2 gene expression and shutdown of early and late viral genes during latent infection of the glioblastoma cell line U138-MG with selectable recombinants of human cytomegalovirus. Virology **204:**101–113.
41. **Yang, N., R. Schule, D. J. Mangelsdorf, and R. M. Evans.** 1991. Characterization of DNA-binding and retinoic acid binding properties of retinoic acid receptor. Proc. Natl. Acad. Sci. USA **88:**3559–3563.
42. **Yu, V. C., C. Delsert, B. Andersen, J. M. Holloway, O. Devary, A. M. Näär, S. Y. Kim, J.-M. Boutin, C. K. Glass, and M. G. Rosenfeld.** 1991. RXRβ: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell **67:**1251–1266.
43. **Zechel, C., X.-Q. Shen, J.-Y. Chen, Z.-P. Chen, P. Chambon, and H. Gronemeyer.** 1994. The dimerization interfaces formed between the DNA binding domains of RXR, RAR and TR determine the binding specificity and polarity of the full-length receptors to direct repeats. EMBO J. **13:**1425–1533.
44. **Zhang, X. K., B. Hoffmann, P. B. V. Tran, G. Graupner, and M. Pfahl.** 1992a. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature (London) **355:**441–446.
45. **Zhang, X. K., J. Lehmann, B. Hoffmann, M. I. Dawson, J. Cameron, G. Graupner, T. Hermann, P. Tran, and M. Pfahl.** 1992b. Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. Nature (London) **358:**587–591.